Glucosamine Sulfate Reduces Prostaglandin E2 Production in Osteoarthritic Chondrocytes Through Inhibition of Microsomal PGE Synthase-1

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ABSTRACT. Objective. Glucosamine sulfate (GS) has been inferred to have a potential antiinflammatory effect on osteoarthritis (OA). We investigated its effect on prostaglandin E2 (PGE2) in human OA chondrocytes, and the level in the PGE2 pathway at which its effect takes place.

Methods. We investigated the effect of GS treatment (0.05, 0.2, 1.0, and 2.0 mM) in OA chondrocytes in the absence or presence of interleukin 1β (IL-1β; 100 pg/ml). We determined the expression levels and protein production/activity of PGE2, cyclooxygenase-1 (COX-1), COX-2, microsomal PGE synthase-1 (mPGES-1), glutathione, and peroxisome proliferator-activated receptor-γ (PPARγ), using specific primers, antibodies, and assays.

Results. GS treatment at 1 and 2 mM significantly inhibited (p ≤ 0.03) production of endogenous and IL-1β-induced PGE2. GS in both the absence and presence of IL-1β did not significantly modulate COX-1 protein production, but GS at 1 and 2 mM demonstrated a decrease in COX-2 glycosylation in that it reduced the molecular mass of COX-2 synthesis. Under IL-1β stimulation, GS significantly inhibited mPGES-1 messenger RNA expression and synthesis at 1 and 2 mM (p ≤ 0.02) as well as the activity of glutathione (p ≤ 0.05) at 2 mM. Finally, in both the absence and presence of IL-1β, PPARγ was significantly induced by GS at 1 and 2 mM (p ≤ 0.03).

Conclusion. Our data document the potential mode of action of GS in reducing the catabolism of OA cartilage. GS inhibits PGE2 synthesis through reduction in the activity of COX-2 and the production and activity of mPGES-1. These findings may, in part, explain the mechanisms by which this drug exerts its positive effect on OA pathophysiology. (First Release Nov 15 2011; J Rheumatol 2012;39:635–44; doi:10.3899/jrheum.110621)

Key Indexing Terms: GLUCOSAMINE SULFATE OSTEOARTHRITIS PROSTAGLANDIN E2 MICROSOMAL PGE SYNTHASE-1 CYCLOOXYGENASE

Osteoarthritis (OA) is the most common form of arthritis and is characterized by degradation and loss of articular cartilage. The hallmark of the disease is the progressive degeneration of articular cartilage and subsequent joint space narrowing. OA leads to pain, loss of motion, instability, and physical disability, thus impairing quality of life. OA results from a complex system of interacting mechanical, biological, biochemical, molecular, and enzymatic/inflammatory feedback loops. The final common pathway is joint tissue destruction resulting from failure of cells to maintain a homeostatic balance between matrix synthesis and degradation. As the disease advances, the degradative process eventually exceeds the anabolic process, leading to progressive joint tissue lesions.

Prostaglandin E2 (PGE2) is implicated in joint inflammation and is among the major catabolic mediators involved in cartilage degradation. OA cartilage spontaneously releases more PGE2 than normal cartilage and knockout mice for a PGE2-specific receptor demonstrated absence of cartilage degradation in a collagen-induced arthritis model. Prostaglandins are biologically active metabolites of arachidonic acid. PGE2 is a well-characterized mediator of inflammation. It contributes to the pathogenesis of arthritis not only by inducing pain, but also by increasing the production of catabolic molecules including proinflammatory cytokines.
kines, matrix metalloproteinases (MMP), and reactive oxygen species, which in turn contribute to articular tissue alterations. It is believed that several of the effects of interleukin-1β (IL-1β) are associated with stimulation of production of PGE₂. In addition to exerting inflammatory actions of its own, PGE₂ can potentiate the effects of other mediators of inflammation.

An anti-inflammatory potential has been inferred from the effect of glucosamine sulfate (GS) through PGE₂, which can explain, at least in part, the significant effect of GS in several short- and longterm clinical trials in OA, some even showing structure-modifying effects in patients with knee OA. This contrasts with another recent report that glucosamine hydrochloride (G.HCl) did not reduce pain effectively in patients with OA. Although G.HCl is not considered effective in contrast to GS when combined with chondroitin sulfate in the latter study, it provided statistically significant pain relief compared with placebo in a subset of patients with moderate to severe knee pain. A possible explanation for this could be that patients with more severe OA experience increased inflammation, which permits different glucosamine preparations to exhibit their effectiveness.

The effect of GS on chondrocytes and cartilage has been investigated primarily in animal studies, with very few studies investigating its effect on human OA cells. In brief, GS was shown to prevent cartilage degradation in animals by acting directly on chondrocytes. It prevented proteoglycan degradation, an effect that is likely secondary to the suppression of aggrecanase and downregulation of aggrecanase. It also downregulates members of the metalloproteinase family and exerts anti-inflammatory activities by diminishing nitric oxide and PGE₂ levels.

To date, the clinical efficacy of drugs targeting inhibition of PGE₂ has been limited to the inhibition of activities of cyclooxygenase (COX)-1 and COX-2 (nonsteroidal anti-inflammatory drugs; NSAID) or COX-2 selective inhibitors (COXIB). Although COXIB were associated with reduced incidence of the serious gastrointestinal side effects observed with conventional NSAID, these drugs demonstrated increased risk of cardiovascular events associated with inhibition of COX-2. For this reason, rofecoxib was withdrawn worldwide in late 2004, followed by valdecoxib in 2005. This withdrawal has raised several serious questions regarding the balance of safety and efficacy of this class of drug and as a result, alternative methods for PGE₂ inhibition with no serious side effects are critically needed; GS is potentially one such compound.

We investigated, in OA chondrocytes, the effect of GS treatment on the level of PGE₂ as well as on precursor enzymes responsible for its production.
(1:2000; all from Cayman Chemical, Ann Arbor, MI, USA), and a rabbit anti-human GAPDH (1:50,000; Abcam, Cambridge, MA, USA) overnight at 4°C. The membranes were washed with TTBS 1× and incubated 1 h at room temperature with the second antibody (COX-1 and COX-2, 1:20,000 anti-mouse; mPGES-1, 1:10,000 anti-rabbit; GAPDH, 1:50,000 horseradish peroxidase-conjugated anti-rabbit IgG; Pierce Chemical, Rockford, IL, USA) and washed again with TTBS 1×. Detection was performed by chemiluminescence using the Super Signal West Dura extended duration substrate (Pierce) and exposure to Kodak Biomax photographic film. Band intensity was measured by densitometry using TotalLab TL 100 software and data were expressed as arbitrary units of the ratio of the target protein/GAPDH relative to the control, which was assigned the value of 1.

Other determinations. The level of PGE₂ was determined in the culture medium and quantified by a specific enzyme immunoassay and that of the glutathione was determined in the cell lysate and quantified by an assay kit (both from Cayman Chemical). All determinations were performed in duplicate for each cell culture.

Statistical analysis. Data are expressed as the mean ± SEM of independent specimens. Statistical significance was assessed by Student’s unpaired or paired t test when appropriate.

RESULTS

Effect of GS on levels of PGE₂ production in OA chondrocytes. To determine whether GS plays a role in the PGE₂ production in cartilage during the OA process, we investigated the effect of GS on this factor, and further, at which level it acted in the PGE2 pathway. Data showed that in OA chondrocytes, the basal PGE₂ production was low and that cells stimulated with IL-1β exhibited a significant increase (p ≤ 0.0001). Interestingly, in both the absence and presence of IL-1β, GS dose-dependently inhibited PGE₂ production, and significance was reached at 0.2 mM for the endogenous and at 1 mM for IL-1β-induced production of PGE₂ (Figure 1).

Effect of GS on expression of COX-1, COX-2, and mPGES-1 in OA chondrocytes. Since COX-1, COX-2, and mPGES-1 are the major enzymes involved in production of PGE₂, we next examined at which level the effect of GS was attributable. We investigated the effect of GS on the expression pattern of COX-1, COX-2, mPGES-1, and related factors in OA chondrocytes.

Data first revealed that COX-1 mRNA expression was decreased by about 67% (p ≤ 0.0001) by IL-1β in OA chondrocytes. GS had no effect on COX-1 mRNA expression in either the absence or presence of IL-1β (Figure 2A, 2B). At the protein level, although significance was not reached, a numerical decrease in the level of COX-1 production under both endogenous and IL-1β-stimulated cell conditions was observed with GS at a concentration of 2 mM (Figure 2C, 2D).

As expected, the level of COX-2 expression was significantly stimulated by IL-1β (15-fold; p ≤ 0.05), and GS did not significantly affect its mRNA level (Figure 3A, 3B). As for protein production, data showed that at lower concentrations the molecular mass of COX-2 is 72–74 kDa. However, at 2 mM in the absence of IL-1β and at 1 and 2 mM in the presence of IL-1β, the apparent molecular mass decreased to 66–70 kDa, and in the presence of IL-1β at 1 and 2 mM, there was a strong accumulation of COX-2 (Figure 3C, 3D). These data are in accord with findings reported for G.HCl in other human cell types28, and indicate that in human OA chondrocytes, GS prevents COX-2 cotranslational N-glycosylation.

Further experiments were performed downstream of the COX, and we investigated the effect of GS treatment on the expression of mPGES-1, the key terminal synthase responsi-

![Figure 1](https://www.jrheum.org/)

**Figure 1.** Effect of glucosamine sulfate (GS) on human OA chondrocyte production of prostaglandin E₂ (PGE₂) in (A) the absence (n = 6) and (B) presence (n = 7–10) of interleukin 1β (IL-1β; 100 ng/ml). Cells were treated for 72 h in the absence [control (C)] or presence of GS at 0.05, 0.2, 1, and 2 mM. At the end of the incubation period, the culture medium was removed and processed for PGE₂ determination. Data are expressed as mean ± SEM and statistical significance was assessed by Student’s t test; p values are versus the control group. Of note, statistical significance was achieved (p ≤ 0.0001) between the control in the absence of IL-1β and the control in the presence of IL-1β.
ble for the biosynthesis of the inducible PGE\textsubscript{2}. As with PGE\textsubscript{2}, the endogenous expression and production of mPGES-1 were low, and IL-1\(\beta\) elicited a 5- and 2.3-fold increase (\(p \leq 0.05\)) in levels of mRNA and protein, respectively. GS significantly inhibited the levels of both mPGES-1 mRNA and protein at 2 mM in the absence of IL-1\(\beta\) and at 1 and 2 mM under IL-1\(\beta\) stimulation (Figure 4).

**Effect of GS on glutathione production in OA chondrocytes.** It is well established that mPGES-1 requires glutathione as an essential cofactor for its activity. Additional experiments were carried out to verify if the GS-mediated decrease in mPGES-1 production was related to alterations in glutathione production in OA chondrocytes. Data showed that IL-1\(\beta\) did not modulate glutathione production, but it was modestly yet significantly reduced (\(p \leq 0.05\)) by GS at 2 mM concentration (Figure 5A, 5B).

**Effect of GS on PPAR\(\gamma\) expression in OA chondrocytes.** It is also well known that PPAR\(\gamma\) activation can inhibit the IL-1\(\beta\)-mediated induction of mPGES-1\textsuperscript{29}. We therefore investigated whether the GS-induced inhibition of mPGES-1 correlated with an increase in the levels of PPAR\(\gamma\). IL-1\(\beta\) markedly decreased (\(p \leq 0.003\)) PPAR\(\gamma\) mRNA expression. As with mPGES-1, in both the absence and presence of IL-1\(\beta\), GS induced a significant increase in PPAR\(\gamma\) expression in a dose-dependent fashion in OA chondrocytes (Figure 6A, 6B).

**DISCUSSION**

Increased production of PGE\textsubscript{2} is a key event associated with the pathogenesis of OA, and inhibitors of PGE\textsubscript{2} biosynthesis are increasingly used for the treatment of this disease and other conditions associated with elevated levels of PGE\textsubscript{2}. The induced synthesis of PGE\textsubscript{2} requires 2 rate-limiting enzymes within the arachidonic acid metabolic pathway:
COX-2 and mPGES-1. In our study, GS suppressed IL-1ß-induced PGE2 production that correlates with an inactivation of COX-2 as well as a suppression of the IL-1ß-induced production of mPGES-1 and the activity of its cofactor, glutathione. In addition, GS enhanced the expression of PPARγ, a potent antiinflammatory factor known to inhibit the IL-1ß-mediated induction of mPGES-1.

Glucosamine is a naturally occurring amino monosaccharide that has been shown to exhibit protective properties in OA joint tissues and in other arthritic diseases in humans and animals. However, the mechanism of action of GS remains unclear.

We observed that GS dose-dependently inhibited IL-1ß-induced PGE2 production but that it was not related to COX-2 mRNA levels. This is in agreement with the findings of Jang, et al, who demonstrated that GS has no effect on the steady state of COX-2 mRNA or the newly synthesizing COX-2 mRNA in IL-1ß-treated A549 cells. However, this is in contrast to other studies showing that GS suppressed COX-2 expression at the transcriptional level. For instance, Rafi, et al reported that G.HCl transcriptionally prevented lipopolysaccharide-induced COX-2 expression by inhibiting NF-κB in murine macrophages. Moreover, Largo, et al showed that treatment with GS suppressed IL-1ß-induced COX-2 expression through transcriptional downregulation and suppression of NF-κB DNA binding activity in human chondrocytes, and as well, decreased COX-2 expression on peripheral blood mononuclear cells in rabbits with atherosclerosis aggravated by chronic arthritis. The reasons for these discrepancies are unclear but may relate to differences in the experimental conditions, cell species, and the glucosamine preparation. In this context, Rafi, et al used G.HCl, the effect of which may differ from that of GS.

With regard to COX-2 protein our data revealed that IL-1ß induced a strong expression of COX-2 protein, and treatment with GS caused a decrease in the COX-2 protein molecular mass. Interestingly, the appearance of the COX-2 with the low molecular mass coincides with the reduction in

![Figure 3. Effect of glucosamine sulfate (GS) on human osteoarthritis chondrocyte (A, B) gene expression of COX-2 (n = 6) and (C, D) protein level (n = 6) as assessed by Western blot in the absence (A, C) and presence (B, D) of interleukin 1β (IL-1β; 100 ng/ml). Cells were treated for 18 h (A, B) or 72 h (C, D) in the absence [control (C)] or presence of GS at 0.05, 0.2, 1, and 2 mM. At the end of the incubation period (A, B), total RNA was extracted and processed for real-time polymerase chain reaction or (C, D) cells were released and processed for Western blot. Data (A, B) were calculated over the housekeeping gene RNA polymerase II and expressed as mean ± SEM of arbitrary unit over the control, which was attributed a value of 1. Statistical significance was assessed by Student’s t test. mRNA: messenger RNA; COX-2: cyclooxygenase-2.](https://www.jrheum.org)
PGE\(_2\) synthesis. COX-2 is an N-glycoprotein with 4 glycosylation sites\(^{33}\), and it has been shown that inhibition of COX-2 N-glycosylation by site-directed mutagenesis or pharmacologically using an N-glycosylation inhibitor leads to the production of a COX-2 protein with reduced molecular mass and activity\(^{34}\). Therefore, our data suggest that GS suppresses IL-1\(\beta\)-induced PGE\(_2\) production by inhibiting COX-2 N-glycosylation and this factor’s subsequent inactivation. This is supported by recent findings that G.HCl prevented the induction of PGE\(_2\) production by IL-1\(\beta\), tumor necrosis factor-\(\alpha\), or phorbol 12-myristate 13-acetate through inhibition of COX-2 glycosylation in human skin fibroblasts\(^{35}\), and in the AS49 human lung epithelial cells\(^{28}\).

In addition to COX-2, mPGES-1 is a rate-limiting enzyme downstream of COX enzymes and specifically converts prostaglandin H\(_2\) to PGE\(_2\). mPGES-1 is coupled with COX-2 and catalyzes the terminal step in the biosynthesis of PGE\(_2\). We found that GS suppressed both basal and IL-1\(\beta\)-induced mPGES-1 expression and protein levels. These data thus suggest that GS can suppress PGE\(_2\) biosynthesis not only through modulation of COX-2 activity, but also by suppressing mPGES-1 expression. This concurs with data in which treatment with GS suppresses IL-1\(\beta\)-induced mPGES-1 expression in bovine articular cartilage explants and in equine chondrocytes and synoviocytes\(^{21,36,37}\). Further, our data also showed that GS inhibits the activity of glutathione, an essential cofactor for mPGES-1 activity. Therefore, in addition to its effects on COX-2, GS can also suppress PGE\(_2\) biosynthesis by inhibiting mPGES-1 production and its activity.

Several lines of evidence suggest that PPAR\(\gamma\) activation may have therapeutic benefits in OA and possibly in other chronic articular diseases. PPAR\(\gamma\) activation suppressed the expression of several genes considered essential in the pathogenesis of OA, including IL-1\(\beta\), inducible nitric oxide synthase, MMP-1, and MMP-13, and prevented the

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**Figure 4.** Effect of glucosamine sulfate (GS) on human osteoarthritis chondrocyte (A, B) gene expression of mPGES-1 (n = 5–8) and (C, D) protein level (n = 5–8) assessed by Western blot in (A, C) the absence and (B, D) presence of interleukin 18 (IL-1\(\beta\); 100 ng/ml). Cells were treated for (A, B) 18 h or (C, D) 72 h in the absence [control (C)] or presence of GS at 0.05, 0.2, 1, and 2 mM. At the end of the incubation period (A, B), total RNA was extracted and processed for real-time polymerase chain reaction or (C, D) cells were released and processed for Western blot. Data were calculated over the housekeeping gene RNA polymerase II or protein GAPDH and expressed as mean ± SEM of arbitrary unit over the control, which was attributed a value of 1. Statistical significance was assessed by Student’s t test. mRNA: messenger RNA.
IL-1β-induced proteoglycan degradation. PPARγ activation was also shown to inhibit PGE₂ production by suppressing the expression of COX-2 and mPGES-1. Other studies in animal models of OA demonstrated the protective effect of PPARγ on the diseased articular tissues. Therefore, it was logical to speculate that GS-mediated suppression of PGE₂ production could also be regulated by modulation of expression of PPARγ. In that regard, data from our study showed that GS enhanced PPARγ expression in OA chondrocytes, suggesting that it may suppress PGE₂ biosynthesis indirectly through upregulation of expression of PPARγ.

A limitation of our study that is inherent to in vitro...
ies is that the concentrations used for GS may represent supraphysiological therapeutic levels of the drug. The concentrations were chosen according to the most current literature and are within the range of in vitro concentrations used by the majority of investigators. The glucosamine concentration found in plasma and synovial fluid after oral 1500 mg GS in humans is 10 µM. In the literature, such low concentration demonstrated no effect and generally the lowest effective concentration reported was not < 50 µM. However, these experiments were performed on healthy bovine cartilage explants and generally using a long incubation period (14–28 days). Of note, it has been reported that higher concentrations of glucosamine should be used when experiments are performed on OA chondrocytes compared to normal chondrocytes\(^{20}\). Another interesting point is that comparison between experiments done between the culture medium containing 10% serum and serum-free revealed that for the latter condition, glucosamine is much less effective\(^{41}\). This effect was suggested to be related to the presence of factors (e.g., growth factors) in the serum that could contribute to the response. In our current work, experiments were performed with 0.5% serum, which is a minimal concentration generally used to avoid having a biased effect. In addition, previous studies performed with the radiolabeled molecule have shown that glucosamine tends to concentrate in the cartilage, where concentrations may increase with repeated administrations\(^{42}\), and may be much higher than those found in plasma and synovial fluid after administration of therapeutic doses\(^{43,44}\). Of importance also is that the pharmacokinetics of glucosamine is modulated by the levels of glucose in the culture medium, as it uses glucose transporters to be taken up by the cells\(^{45,46}\). Because our cells are grown in a medium with high levels of glucose (DMEM containing 25 mM glucose), it was necessary to use high concentrations of glucosamine in order to appreciate its effect.

In addition, it should be noted that treatment with GS is characterized by a slow onset of action, with a maximal clinical effect being attained after several months, i.e., 3 to 6 months. Hence, in order to reproduce in vitro an effect observed in vivo obtained after several weeks of treatment, it appears to be necessary to increase the drug concentrations for in vitro determination.

Another limitation is that some could argue that in in vitro experiments the GS will dissociate in the culture medium and increase the level of free sulfate concentration, which could influence the results. In fact, it has been shown that the serum level of inorganic sulfate increases after the administration of GS\(^{47}\), and one could hypothesize that it may contribute to the pharmacologic effect of the drug. Such a hypothesis could be supported by recent reports on the in vitro comparison between GS and G.HCl\(^{48,49}\) on cartilage catabolic and anabolic factors showing that both preparations demonstrated similar outcomes, but that GS was more effective. Moreover, although there has not been a head to head clinical study between these preparations of glucosamine (GS and G.HCl), trials showed that GS\(^7,8,9,10,11\) in contrast to G.HCl\(^{12,50}\) was effective, in addition to having better pharmacokinetics\(^{23}\).

We have demonstrated that GS inhibits IL-1β-induced production of PG\(_E2\) in human OA chondrocytes, and that the inhibition appears to occur at multiple levels: induction of a shift in the molecular mass of COX-2, and thus its inactivating inhibition; of mPGES-1 expression and production; and augmentation of expression of PPAR\(_γ\). These findings could at least in part explain the protective effects of GS in OA and other inflammatory arthritides.

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